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Studies are directed to the chemical structure of acetylcholinesterase, with particular reference to the positions and identification of amino acid residues involved in catalysis, inhibitor binding, secretion and linkage to structural subunits. This work is dependent on the known primary structure of acetylcholinesterase and involves the use of site-directed covalent inhibitors of the active site and peripheral amionic site, selective antibodies and peptide isolation. The work has been developed to complement ongoing molecular biological studies of enzyme structure and site-specific mutagenesis. (To date, our studies have shown that the catalytic subunits from the asymmetric and glycophospholipid forms of the enzyme diverge at residue 534 in the C-terminus of the molecule. Epitopes to the antibodies 2C-9 and 4E-7 have been tentatively assigned. Using these and other antibodies we have determined the localization of the asymmetric and glycophospholipid containing forms of the enzyme in the synapse.)

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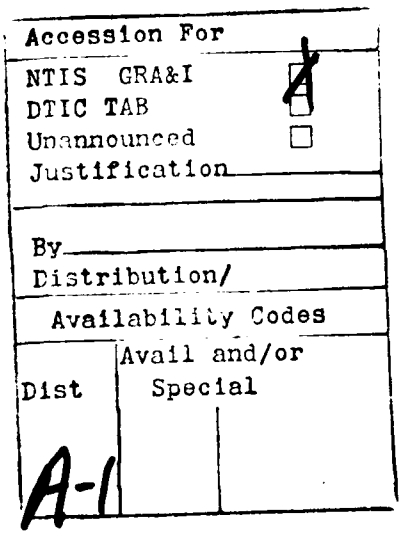


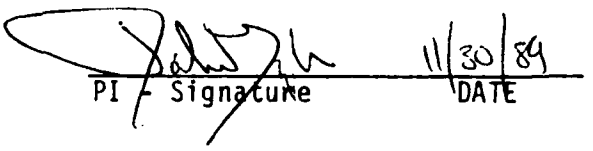


TABLE OF CONTENTS

The Primary Sequence of Acetylcholinesterase and Selective Antibodies for the Detection of Organophosphate Toxicity

List of Figures and Tables

I. INTRODUCTION

Acetylcholinesterase (AChE) was proposed to exist in biological tissue exactly 75 years ago[1] and was characterized as a site of drug action in the early studies of Dale and colleagues, who demonstrated that physostigmine prolonged the action of acetylcholine. Starting in the mid-19th century[2], AChE inhibitors have enjoyed therapeutic applications in the treatment of glaucoma, smooth muscle atony, certain arrhythmias, and myasthenia gravis, and have been used to reverse competitive neuromuscular blockade[3]. Recently, reports, albeit largely anecdotal, of the use of certain AChE inhibitors in the amelioration of Alzheimer's disease have appeared[4]. In addition, these agents have been used widely as agricultural and garden insecticides. Early studies also established that AChE catalysis was typical of serine hydrolases. The pioneering work of Irwin Wilson and his colleagues demonstrated the principle of site direction for developing both selective inhibitors and reactivators of the enzyme[5].

A critical direction in the study of the structure of the AChE's was set with the finding of Massoulié and Reiger[6] that a synaptic form of AChE was dimensionally asymmetric and linked to a filamentous structural subunit. Treatment of these species with proteases or collagenases removed the tail unit without loss of catalytic activity and resulted in a globular tetramer of subunits. It was later established that the tail unit contained a collagen-like composition[7,8]. The diversity of molecular species of AChE has become more complex and it is now appropriate to divide them into two classes (fig. 1).

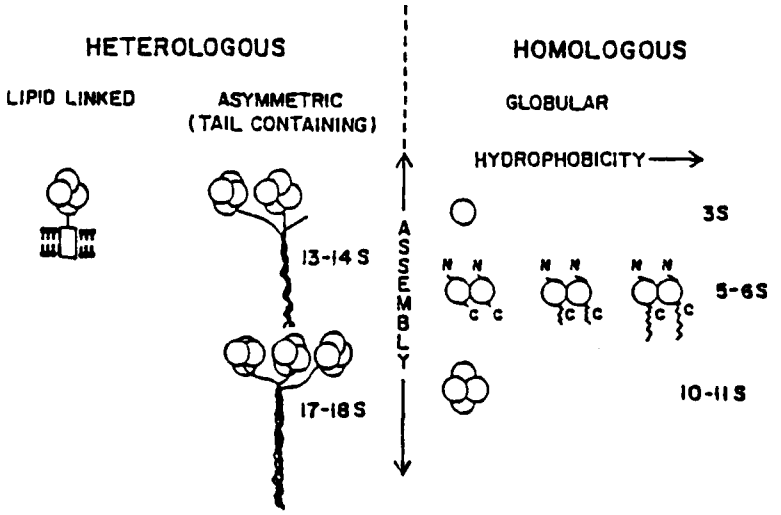


Fig. 1 Polymorphic forms of AChE can be divided into two classes: (1) associations of heterologous subunits (Included in this group are dimensionally asymmetric forms which contain multiple catalytic subunits disulfide-linked to a collagen-like tail unit and a lipid-linked subunit), and (2) homologous forms, which contain associations of identical subunits and differ in hydrophobicity by attachment of a glycophospholipid.

The heterologous class contains catalytic subunits disulfide-linked to structural subunits. It includes the asymmetric, collagen-containing species (designated 'A' forms) found largely in the neuromuscular junction and ganglia[7,8] and a second species, found in brain, in which a tetramer of catalytic subunits is disulfide-bonded to a lipid-linked subunit[9]. The second class consists of species with a homologous association of subunits. Those that are membrane-associated contain a glycophospholipid moiety covalently linked to the C-terminal carboxyl residue in the protein [designated 'H' (for hydrophobic) forms][10,11]. Hence, the membrane-associated forms have distinctive means for tethering themselves to the outside surface of the cell. The expression of a particular molecular species of AChE is under the control of phenomena related to cellular excitability, such as intracellular [Ca^{++}], synaptogenesis, and the formation of action potentials.

Enzyme Structure

AChE was first cloned in 1986 from Torpedo[12]. Since that time the Drosophila cholinesterase sequence and a human butyrylcholinesterase (BuChE) have been reported[13,14]. To date no full-length clones of a mammalian AChE have been described, although several groups of researchers have been making progress towards this end. The AChE sequence defined a new family of serine hydrolases distinct from the pancreatic and Subtilisin families of hydrolases[12] (fig. 2).

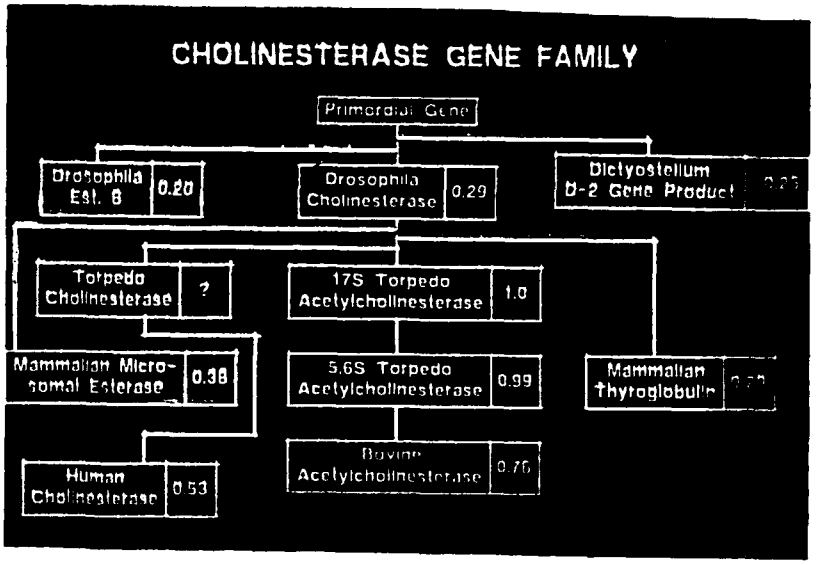


Fig. 2 Cholinesterase Gene Family. Sequence identities come from published sequences (refs. 12–18) and data for bovine AChE are from B.P. Doctor and reflect ~85% of the sequence (unpublished). Lengths of the lines are arbitrary and do not represent a statistical evolutionary tree. The sequence of the 17S AChE is the frame of reference.

Included with this functionally eclectic family are thyroglobulin, a Dictyostelium protein of unknown function, and esterases from the male reproductive organ of Drosophila and from the endoplasmic reticulum of mammalian liver. Moreover, all of the cholinesterases maintain close sequence identity. Analysis of the disulfide bonding pattern in AChE reveals that of the eight cysteines, six are conserved in the three intrasubunit linkages and one (C–231) is free, while C–572 is involved in intersubunit disulfide bonding[19]. That the six cysteines involved in intrasubunit bonding are all conserved in the large gene family indicates that all members have identical folding patterns. The inferred cDNA sequence also reveals a 21–amino–acid–leader peptide but no other obvious membrane spanning regions. Hence, the encoded sequence is targeted for secretion from the cell, and post-translational modifications are responsible for the membrane associations seen in situ.

Genomic Organization

Despite their extensive diversity in structure, all AChE's in vertebrates appear to be encoded by a single gene, with alternative mRNA processing forming the basis of structural polymorphism. The evidence for these considerations is: (a) complete sequence identity of the two forms of the Torpedo enzyme through Thr–535, where a sequence divergence is found[20]. The form containing the glycophospholipid contains only a unique dipeptide sequence (Ala,Cys), to which the glycophospholipid is attached to the terminal Cys. The asymmetric form continues for 40 residues after the divergence point); (b) that RNAse digestion experiments show the single mRNA divergence in the open reading frame to be in the very region encoding the amino acid divergence[21]; (c) isolation of a partial-length cDNA encoding the divergent sequence in the mature protein, plus a processed sequence of 24 amino acids[22]; (d) characterization of a genomic sequence containing the alternative exons

encoding the asymmetric species and hydrophobic species[21,23]; (e) that a synthetic cDNA constructed from the appropriate exons from genomic clones, upon transfection, yields an active membrane-associated enzyme (the expressed enzyme is released upon phospholipase C treatment [cf section C]).

The components of catalytic function (Ser_{200}, His_{425} and/or His_{440} (and, perhaps, anticipated Asp or Glu) in the putative charge-relay system are found in the first exon of the open reading frame, as are the first two disulfide loops. The second exon closes the last disulfide loop, while the two alternatively spliced third exons are responsible for the differential membrane localizations.

Regulation of Acetylcholinesterase Expression

At present, little is known about the regulation of expression of this enzyme, although descriptive studies indicate it is tightly controlled by cellular excitability and intracellular Ca^{++} [24-26]. The asymmetric species does not appear until synaptogenesis ensues and action potentials are seen in muscle. Alternative exon usage involves mRNA processing from nuclear pre-RNA. Presumably, addition of the glycophospholipid presumably occurs cotranslationally, and oligosaccharide processing and the linkage of the catalytic and structural subunits occur at the Golgi stage[25]. Little is known about factors affecting transcription, mRNA stability, or processing; this subject is now appropriate for study. Other studies reveal that AChE is retained in the basal lamina after destruction of the nerve or muscle cell[26-29]. This region serves as a template for new synapse formation upon nerve regeneration.

Studies in our Laboratory

Our laboratory has been engaged in the study of acetylcholinesterase structure for the past 15 years, using approaches intrinsic to protein chemistry and molecular biology. These studies have yielded the first primary[12] and secondary structure[19] of the enzyme. More recently, we have delineated the structure of the gene encoding the enzyme from Torpedo[23] and have established that alternative mRNA processing is responsible for the structural diversity in the acetylcholinesterases[21,23]. Our current studies have been directed to the murine and human acetylcholinesterases for the purpose of examining comparative structures of the enzyme and regulation of gene expression. The finding of a genetic mutation in man which may be related to the splicing mechanism has also allowed us to begin to study acetylcholinesterase structure in man.

II. BODY

A. METHODS

1. Analysis of Sequence of 5.6S Acetylcholinesterase

Digestion of 5.6S Acetylcholinesterase with Phosphatidylinositol-Specific Phospholipase C - Purified 5.6S-AChE (20-60 mg) (1.5-2.0 mg/ml) was radiolabeled by a 1.25-M excess of [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) (specific activity, 10 mCi/mmol) in the presence of 0.02% sodium azide overnight at room temperature. The reaction mixture, after aging 24 h at 4°C, was dialyzed to remove unreacted DFP. The ^{3}H-labeled enzyme was digested with phosphatidylinositol-specific phospholipase C (PI-PLC) purified from either S. aureus (20 μg/ml) or Bacillus thuringiensis (3.5 units/ml) obtained from Martin Low, Columbia University, NY. Digestion was performed in 50 mM Tris, pH 7.2, 2 mM EDTA, 0.1% deoxycholate, 0.02% sodium azide at 37°C for 4-8 h. Digestion by PI-PLC was monitored by sedimentation on sucrose density gradients.

Fractionation of Cysteine-Containing Tryptic Peptides - The PI-PLC digest of 5.6S acetylcholinesterase was dialyzed against 50 mM NH_4HCO_3, pH 8.0, 0.02% sodium azide, and concentrated to ~4 mg/ml by lyophilization. The enzyme was

brought to 6 M in guanidine HCl, adjusted to pH 8 with 1 M Tris base, and incubated 2 h under N_2 at 50°C with a 2-fold M excess of dithiothreitol over estimated total cysteine residues. To label cysteines, [^{14}C]iodoacetate (specific activity, 2-5 mCi/mmol) was added to the reduced, denatured enzyme in 2-fold M excess over total thiols and allowed to react in the dark for 1-2 h at room temperature. A 10-fold excess of dithiothreitol over total thiols was added to quench unreacted iodoacetate. Following dialysis against 50 mM NH_4HCO_3, pH 8.0, the preparation was incubated with trypsin (1% w/w) at 37°C overnight and for another 2 h with additional 1% trypsin. The tryptic peptides were applied to a Sephadex G-50 column (1.5 x 200 cm), equilibrated in 50 mM NH_4OH, and eluted at a flow rate of 20 ml/h. Fractions of 3 ml were collected, monitored for absorbance at 280 nm and for ^{14}C radioactivity, and concentrated by lyophilization to 2 ml. Separation of pooled peptides was by reverse-phase HPLC on Vydac C-4 or C-18 columns in aqueous 0.1% trifluoroacetic acid using an acetonitrile gradient of 0-50% in 180 min and 50-90% in 30 min. Absorbance at 219 nm and ^{14}C radioactivity were monitored.

Further Purification of ^{14}C-Labeled Tryptic Peptides - HPLC fractions containing ^{14}C-peptides unique to the 5.6S species were pooled and further resolved on a Vydac C-4 column in 10 mM phosphate, pH 6.9, using an acetonitrile gradient of 0-50% in 150 min. Fractions containing ^{14}C-peptides were run once more in 0.1% trifluoroacetic acid, using an acetonitrile gradient of 0-12% in 120 min. Peptides that remained unresolved after the initial HPLC fractionation were fractionated by HPLC in phosphate or on a C-18 column in trifluoroacetic acid.

Sequencing and Composition Analysis - Purified ^{14}C-labeled tryptic peptides were sequenced by gas-phase methods, using an Applied Biosystems Protein Sequencer (Model 470A). Aliquots from sequencing steps were counted to determine the sequence position of ^{14}C-carboxymethylated cysteinyl residues. Duplicate peptide samples were hydrolyzed in 6 N HCl at 110°C for 18 h either with prior performic acid oxidation or in the presence of thioglycolic acid alone. Amino acid analysis employed a Kontron Liquimat III amino acid analyzer with postcolumn ortho-phthalaldehyde detection. Glucosamine and ethanolamine contents were determined using an LKB 4400 amino acid analyzer with ninhydrin detection on samples hydrolyzed in vacuo in 6 N HCl at 110°C for 18 h.

Treatment with Glycopeptide N-Glycosidase - Peptide samples were dried under a nitrogen stream and redissolved in 0.1 M sodium phosphate, pH 7, 10 mM EDTA. Digestion with glycopeptide N-glycosidase (ECF 3.2.2.18; 0.2 unit/μl; Boehringer Mannheim) at 37°C for 18 h was followed by reverse-phase HPLC fractionation.

2. Antibody Production, Analysis of Specificity and In Situ Localization

Production of Site-Directed Antibodies - A hexadecapeptide corresponding to the COOH-terminal amino acids (KNQFDHYSRHESCAEL, Lys^{560}-Leu^{575}) of the catalytic subunits of the asymmetric form of acetylcholinesterase was synthesized in the laboratory of Dr. Russell Doolittle (University of California, San Diego) by the Merrifield solid-phase method. The authenticity of the peptide was determined by gas-phase sequencing on a protein sequencer. The peptide was coupled to BSA by slowly adding glutaraldehyde (1 ml, 0.2%) to 2 ml of 100 mM sodium phosphate buffer (pH 7.5) containing 1.5 x 10^{-7} mol (10 mg) of BSA and 7.6 x 10^{-6}mol of peptide. The reaction was allowed to proceed for 30 min at 22°C; then, unreacted glutaraldehyde was quenched by the addition of 0.25 ml of 1.0 M glycine. The result of the coupling reaction was evaluated by SDS-PAGE. The reduced migration of the peptide-BSA conjugate corresponded to an average incorporation of 5-10 mol of peptide per mol of

BSA. In addition, the peptide-BSA conjugate was excised from the gel, and dissolved in 0.5 ml of 1.0 Tris-HCl (pH 7.0). The radioactivity incorporated into the BSA was consistent with an average incorporation of 4-5 mol of peptide per mol of BSA.

Female white New Zealand rabbits (5-6 lbs) were immunized by injection in the isolated lymph nodes of the rear leg and intradermally down the back with 0.4 ml Freund's complete adjuvant. Booster immunizations were performed intradermally after 1 mo, and 50 ml of blood was drawn 2 wk later. The serum was allowed to clot at 22°C, was clarified by centrifugation, and was frozen at -70°C in small aliquots.

SDS-Page and Western Blots - Proteins were mixed with an equal volume of buffer containing 30 mM Tris-HCl (pH 6.8), 1.0% SDS, 5% glycerol, 10 mM DTT, 0.05 mg/ml bromphenol blue, and 0.05 mg/ml pyronin Y. Samples were boiled for 3 min, and proteins were separated by discontinuous SDS-PAGE in 1.5-mm slab gels composed of a constant ratio of acrylamide and N,N'-methylene-bisacrylamide (37:1) polymerized with ammonium persulfate (0.75 mg/ml) and N,N,N'N',-tetramethylethylenediamine (0.67 μl/ml). The stacking gel was 3.3% acrylamide in 25 mM Tris-HCl (pH 6.8), 0.2% SDS, and the separating gel was either 8% or 10% acrylamide in 75 mM Tris-HCl (pH 8.6), 0.2% SDS. The gels were run in a slab gel apparatus (model SE 500; Bio-Rad Laboratories) at 120 V constant voltage in 25 mM Tris, 190 mM glycine (pH 8.6), 0.1% SDS. Proteins were detected in the gels by staining and destaining in the presence and absence, respectively, of Coomassie brilliant blue R (0.15 mg/ml) in 50% methanol, 10% acetic acid.

Electrophoretic transfer (50 V, 150 mA, 4°C, 10-16 H) of proteins from unstained gels to nitrocellulose was performed in a transphor unit after soaking the gel in the transfer buffer (25 mM tris, 190 mM glycine, pH 8.6, 20% methanol) for 30 min. Blotted proteins were detected by staining and destaining in 50% methanol, 10% acetic acid, in the presence or absence, respectively, of Amido black (0.1 mg/ml). Immunodetection of blotted proteins was performed using a Vectastain ABC kit that uses a biotin-labeled goat anti-rabbit antibody and peroxidase-coupled avidin.

Deglycosylation of Acetylcholinesterase - The deglycosylation reactions used ^{3}H-DFP-labeled acetylcholinesterase that had been desalted on a size exclusion column and then lyophilized. ^{3}H-DFP-labeled acetylcholinesterase (1.09 mg) was resuspended in 50 mM sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 1 mM PMSF, 10 μM pepstatin A, 0.5% NP-40, 0.5% β-mercaptoethanol, 0.1% SDS, and digested with endoglycosidase F (20 U) by incubation for 8 hr at 37°C. ^{3}H-DFP-acetylcholinesterase (1.76 mg) was deglycosylated with glycopeptidase F (1.0 U) by incubation in 1.1 ml of 250 mM sodium phosphate buffer (pH 7.4) containing 10 mM EDTA, 1.0 mM EDTA, 1.0 mM PMSF, 10 μM pepstatin A, 0.8% NP-40, 10 mM β-mercaptoethanol, and 0.5% SDS for 18 h at 37°C; [^{3}H]DFP-labeled acetylcholinesterase (2 mg) was treated for 8 h at 37°C with endoglycosidase H (1.0 U) in 0.1 M sodium citrate buffer (pH 5.5) containing 1.0 mM PMSF, 10 mM pepstatin A, 2% SDS, and 1.0% β-mercaptoethanol.

Immunoprecipitation of Acetylcholinesterase - [^{3}H]DFP-labeled acetylcholinesterase (0.4 μg) was incubated with the indicated antibodies in 200 ml of buffer A containing 20 mM sodium phosphate (pH 7.4), 150 nM NaCl, 0.02% NaN_3, 0.01% Tween-20, and 0.1% BSA for 2 h at 37°C. Pansorbin was washed with buffer A containing 5 mM b-mercaptoethanol and 0.5% NP-40, then washed with buffer A containing 0.05% NP-40, and finally resuspended to 2.5% (w/vol) in buffer A. Antibodies were precipitated by the addition of 800 μl of Pansorbin (15 min at 4°C). Precipitates were sedimented by centrifugation in a microfuge, the supernates aspirated, and the pellets resuspended by incubation with 200 μl of 2% SDS and 4 M urea at 90°C for 2 min. The Pansorbin was separated

from the solubilized acetylcholinesterase by centrifugation in a microfuge, and the supernatant was removed for determination of radioactivity. Precipitation of [^{3}H]DFP-labeled acetylcholinesterase by the antibodies was compared to the maximal precipitation obtained by the addition of 1.0 ml of acetone rather than Pansorbin.

Preparation of Tissue for Light and Electron Microscopy - The electric organs were removed from an adult male Torpedo and fixed in 4% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. The tissue was trimmed down to ~3 x 2-mm pieces and cryoprotected in 1.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 30 min at 4°C followed by cryoprotection in 2.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. Tissue was mounted on aluminum specimen support pin so as to provide a cross-section of the electrocytes; it was then frozen in liquid propane cooled with liquid nitrogen.

Conventional electron microscopy was performed, using small pieces of electric organ that were first fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS for 1 h, followed by 1% osmium tetroxide for 1 h. After dehydration with ethanol, the tissue was embedded in EPON-Araldite resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Immunofluorescent Localization of Acetylcholinesterase - Thick sections (2-μm) of frozen tissue were cut on an ultracryomicrotome at -80°C and mounted on clean glass slides, using a drop of 2.0 M sucrose in a platinum loop. Sections were re-equilibrated with 0.1 M PBS for 5 min followed by 0.1 M PBS with 0.05 M glycine for 5 min. To minimize nonspecific staining, the sections were pretreated with 1% gelatin in PBS for 10 min followed by 2% normal goat sera in PBS for 10 min. Sections were incubated in specific antisera or normal rabbit or normal mouse sera for 30 min at room temperature. Dilutions of primary antibodies were 80-B (100x), 2C-9 (100x), 4F-3 (50x), CT (100x), 4E-7 (100x), and antireceptor antibodies (100x). After extensive washing in PBS, the sections were treated with either goat anti-rabbit rhodamine or goat anti-mouse fluorescein conjugate for 20 min. The sections were again washed extensively in PBS, covered with 90% glycerol in PBS, and placed under a coverslip. Sections were examined, using a Leitz 63 x objective on a Zeiss universal microscope.

Electron Microscopic Localization of Acetylcholinesterase - Thin cryosections (0.1 nm) or thick cryosections (0.5 μm) were cut at -100°C and mounted on Formvar filmed, carbon-stabilized gold grids. The thin sections were treated much like the thick sections, except that after incubation in primary antisera, the sections were immunolabeled with goat anti-rabbit or goat anti-mouse IgC conjugated to either 5, 10, 15 or 30 nm gold (Janssen Life Sciences, Piscataway, NJ) for 20 min. After extensive washing in PBS, the sections were fixed in 1% glutaraldehyde/1% osmium tetroxide in PBS for 3 min. Sections were washed in distilled water and counterstained in 2% aqueous uranyl acetate for 30 min. The sections were subsequently dehydrated in ethanol and embedded in a thin film of LR white acrylic resin and polymerized as previously described[24]. Thin cryosections were viewed at 100 KeV with a JEOL 100CX electron microscope, and thick cryosections were viewed at 1 MeV with a JEM 1,000 high-voltage electron microscope.

Studies of Antibody Specificity - Polyclonal antibodies and monoclonal antibodies were raised to the C-terminal and an active center peptide (KTVTIFGESAGGASVGMHILSPGSR). The monoclonal antibodies were raised in B.P. Doctor's laboratory at Walter Reed (WRAIR). Immunoprecipitation and ELISA assays employed procedures developed jointly by the two laboratories (cf 30). Similar assays were also developed to examine antigenicity of tryptic and CNBr peptides from acetylcholinesterase.

B. RESULTS

Sequence Divergence in the Molecular Forms of Acetylcholinesterase - A unique COOH-terminal tryptic peptide from the hydrophobic globular (5.6S) form of Torpedo californica acetylcholinesterase that exhibits divergence in amino acid sequence from the catalytic subunit of the dimensionally asymmetric (17S + 13S) species of the enzyme was identified. The hydrophobic form contains an attached glycophospholipid, and the peptide could be recovered only after treatment with phosphatidylinositol-specific phospholipase C. After reduction, carboxymethylation with [^{14}C]iodoacetate, and trypsin digestion, the resulting peptides were purified by gel filtration and high performance liquid chromatography (HPLC). The HPLC profiles of the labeled cysteine peptides from the 5.6S enzyme revealed a unique radioactive peak which was not present in digests of the asymmetric form. This radioactive peptide, which had been excluded on Sephadex G-50, eluted early as a broad peak on HPLC. The peak contained sufficient ^{14}C-radioactivity to account for a single cysteine, but had an unusually low extinction at 219 nm for one equivalent of excluded peptide. Further HPLC purification generated multiple peaks, all of which yielded identical amino acid sequences. The difference in chromatographic behavior of the individual peaks most likely reflects heterogeneity in post-translational processing. Gas-phase sequencing and composition analysis are consistent with the sequence: Leu-Leu-Asn-Ala-Thr-Ala-Cys. The composition includes 2-3 mol each of glucosamine and ethanolamine -- which is indicative of modification by glycophospholipid. Glucosamine is also present in an asparagine-linked oligosaccharide. The two forms of acetylcholinesterase diverge after the Thr residue of this peptide; the peptide chain of the hydrophobic form terminates after cysteine, whereas the asymmetric form continues for 40 amino acids beyond the divergence. The locus of the divergence and absence of other sequence differences between the two forms suggest that the molecular forms of acetylcholinesterase arise from a single gene by alternative mRNA processing.

Antibodies to Acetylcholinesterase; Their Use in In Situ Localization - Table 1 summarizes antibodies in use for the study of the localization of acetylcholinesterase.

Table 1. *Staining intensity of antibodies used in immunocytochemical localization of acetylcholinesterase in Torpedo electric organ.*

Antigen	Name	Species	Poly/Mono	Subtype	Light	EMperox*	EMgold§	Form@
11 S	80A	Rabbit	Polyclonal	-	+++	+++	++	Both
11 S	80B	Rabbit	Polyclonal	-	++	++	++	Both
17S,5.6S	4E7	Mouse	Monoclonal	IgG2b	+++	++	+	5.6 S
17S,5.6S	4F3	Mouse	Monoclonal	IgG1	++	+	+	17 S
11S	2C9	Mouse	Monoclonal	IgG1	+++	ND	+++	Both
11S	2C6	Mouse	Monoclonal	IgG1	+++	++	+	ND
Peptide$	CT	Rabbit	Polyclonal	-	+++	++	++	17 S
Peptide#	AS	Rabbit	Polyclonal	-	-	ND	ND	Both

(*) Represents results obtained with the electron microscope and peroxidase-labeled secondary antibodies.

(§) Represents results obtained with the electron microscope and gold-labeled secondary antibodies.

(@) The molecular form of acetylcholinesterase (5.6 S, 17 S, or both) recognized by the antibody is indicated.

($) The synthetic peptide Lys^{560}-Leu^{575}, corresponding to the C-terminal amino acids of the catalytic subunit of the asymmetric form of acetylcholinesterase from *Torpedo c.*, was used as the antigen.

(#) The synthetic peptide Lys^{192}-Arg^{216}, corresponding to amino acids common to both forms of acetylcholinesterase from *Torpedo c.*, was used as the antigen.

(ND) Not determined.

Sequence-specific antibodies raised against a synthetic peptide corresponding to the COOH-terminal region (Lys^{560}-Leu^{575}) of the catalytic subunits of the asymmetric form of acetylcholinesterase reacted with the asymmetric form of acetylcholinesterase, but not with the hydrophobic form. These results confirm recent studies suggesting that the COOH-terminal domain of the asymmetric form differs from that of the hydrophobic form, and represent the first demonstration of antibodies selective for the catalytic subunits of the asymmetric form. In addition, the reactive epitope of a monoclonal antibody (4E7), previously shown to be selective for the hydrophobic form of acetylcholinesterase, has been identified as an N-linked complex carbohydrate, thus defining posttranslational differences between the two forms. These two form-selective antibodies, as well as panselective polyclonal and monoclonal antibodies, were used in light- and electron-microscopic immunolocalization studies to investigate the distribution of the two forms of acetylcholinesterase in the electric organ of Torpedo. Both forms were localized almost exclusively to the innervated surface of the electrocytes. However, they were differentially distributed along the innervated surface. Specific asymmetric-form immunoreactivity was restricted to areas of synaptic apposition and to the invaginations of the postsynaptic membrane that form the synaptic gutters. In contrast, immunoreactivity attributable to the hydrophobic form was selectively found along the nonsynaptic surface of the nerve terminals and was not observed in the synaptic cleft or in the invaginations of the postsynaptic membrane. This differential distribution suggests that the two forms of acetylcholinesterase may play different roles in regulating the local concentration of acetylcholine in the synapse.

A large part of this study involved fluorescence microscopy and electron microscopy, which is summarized in figures 3, 4 and 5. Antibodies that react with sequence common to both enzyme forms (i.e. the polyclonal antibody 80-B and the monoclonal antibody 2C-9) show staining within the postsynaptic invaginations as well as within two layers of the postsynaptic surface (fig. 3A and B). Antibodies specific for the tail subunit, monoclonal antibody 4F3, and for sequence unique to the catalytic subunit, polyclonal antibody CT, show a single layer of staining which again extends into the postsynaptic folds (fig. 3C and E). Lastly, antibody selective for the glycophospholipid-containing form of the enzyme, monoclonal antibody 4E-1, shows punctate staining on the postsynaptic membrane. Some non-specific staining can be detected on the dorsal non-inverted surface which is likely due to a common carbohydrate epitope. This becomes even more evident at high magnification (fig. 4A, B, C and D). Moreover, the punctate staining of this form of the enzyme can be contrasted for the uniform staining of the membrane seen for the acetylcholine receptor by antibody reactivity (figs. 4E and F).

The above studies with fluorescence microscopy can be carried to the electron microscopy level using colloidal gold (fig. 5A). Again, the antibodies reactive to common sequence are found on the nerve terminus and deep in the post-synaptic folds (fig. 5A). By contrast, antibody directed to the glycophospholipid containing form of the enzyme show staining on only the nerve termini, which likely accounts for the punctate staining seen upon fluorescence microscopy (fig. 5B). These findings point to a nerve cell body origin of the glycophospholipid form of the enzyme in the synapse. Surprisingly, this form of the enzyme is preferentially localized on the non-synaptic surface.

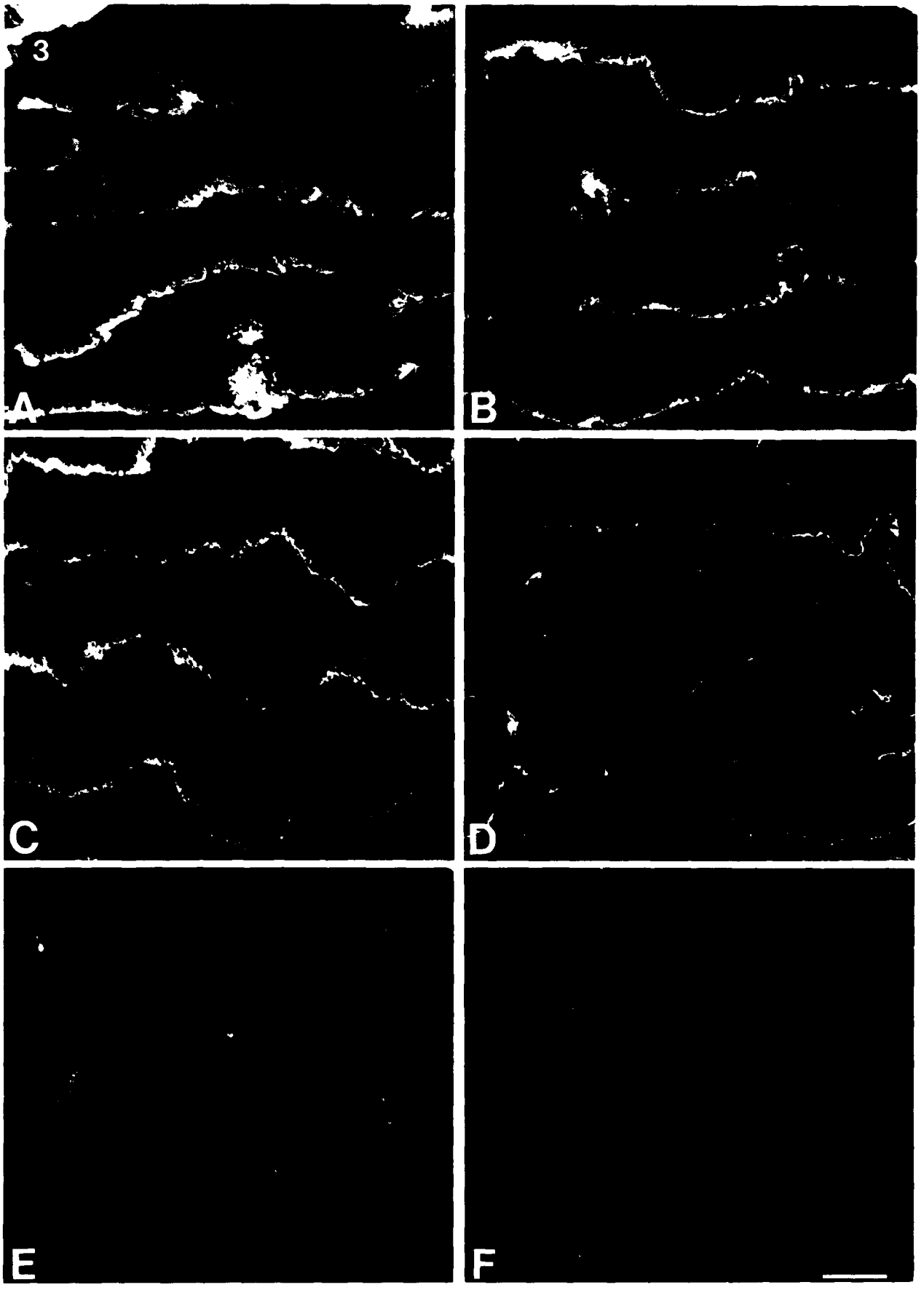
Analysis of Antibody Specificity and Accessibility of Epitopes on Acetylcholinesterase - Polyclonal and monoclonal antibodies were generated against a synthetic peptide (25 amino acid residues) corresponding to the sequence of the active-site-containing region of Torpedo californica acetylcholinesterase

by coupling to bovine serum albumin or encapsulation into liposomes containing lipid A as an adjuvant prior to immunization-produced antibodies of high titer. In order to determine whether the active-site-serine-containing region of AChE is located on the surface of the molecule (and is, thus, accessible for binding to antibodies) or is located in a pocket (and, thus, is not accessible to binding), the immunoreactivity of these antibodies was determined using ELISA, immunoprecipitation, Western blots, and competition ELISA. Both AChEs, Torpedo and fetal bovine serum, failed to react with several of these MAbs in native form, but showed significant cross-reactivity with denatured enzymes. Other antibodies interacted with both the native and denatured form of the enzyme. Human serum BuChE, which has high amino acid sequence homology to these AChEs, failed to react with the same MAbs, either in native form or denatured form. Chymotrypsin also failed to react with these MAbs in either form. The results suggest that the active-site-serine-containing region of these AChEs in native state is not exposed on the surface of the enzyme and is, most likely, located in a crevice-like conformation.

Current studies are under way to ascertain whether the antibodies reactive with the native and denatured form of the enzyme (as opposed to those reactive with the native form) interact with different epitopes on this 25-amino-acid peptide. Initially, this is being pursued by cleavage of the peptide at its single methionine. This work is being done in collaboration with the biochemistry group at WRAIR. Two randomly selected monoclonal antibodies raised against homogeneous 11S AChE, 4E-7 and 2C-9, which were used extensively in the immunocytochemical studies, were characterized in terms of their sequence specificity. 4E-7 reacts with an epitope sensitive to removal of an N-linked oligosaccharide, whereas 2C-9 reacts solely with the peptide backbone. Both have been localized to a peptide extending between residues 44 and 83 containing one N-linked glycosylation site. Future work will be directed to localizing further these respective epitopes.

Identification of the Active Center Surface of the Enzyme and the Sulfhydryl-Group Arrangement for Linkage to Structural Subunits - DFP labeling has shown that Ser 200 is the catalytic serine. Sequences of the cholinesterases show two histidines at 425 and 440 to be conserved. Our site-specific mutagenesis work has shown that residue 440 is the serine involved in the charge-relay system (cf fig. 6), since activity is completely eliminated with the His 440 mutation.

Work is in process to identify other residues with the use of two azido phosphonates which cross-link the active site serine and a residue(s) in the active center. To date we have found a peptide starting at residue 290 cross-linked to an active-site tryptic peptide. These peptides are being characterized further.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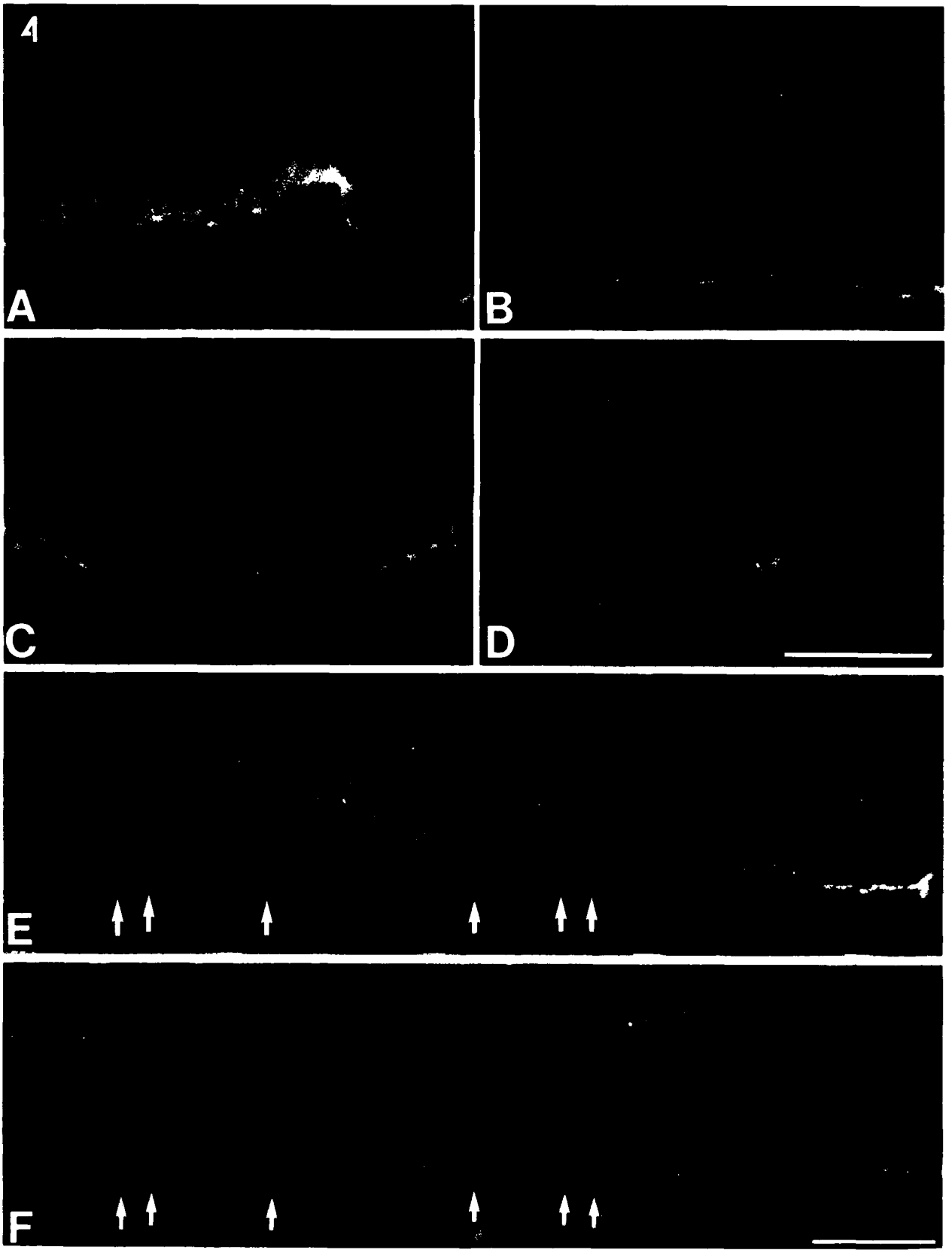
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Fig. 3: Immunofluorescent localization of acetylcholinesterase in *Torpedo* electric organ. Tissue sections were prepared as described in Materials and Methods and were incubated with either (A) 80-B, (B) 2C-9, (C) 4F-3, (D) 4E-7, (E) CT, or (F) CT antibodies blocked by earlier incubation with the COOH-terminal peptide (2.8×10^{-7} M). These primary antibodies were visualized with rhodamine or fluorescein-labeled secondary antibodies, Bar, 10μm.

Fig. 4: High magnification of immunofluorescent staining. The primary antibodies were (A) 2C-9, (B) 4E-7, (C) 4F-3, and (D) CT. Tissue sections were also double labeled with 4E-7 (E) and antibodies selective for the nicotinic acetylcholine receptor (F). The same section is shown in both E and F, where the primary antibodies were visualized with fluorescein and Texas red-labeled secondary antibodies, respectively. The arrows (identical positions in both E and F) indicate nerve terminals stained by 4E-7 (E) that lie in shallow troughs in the postsynaptic membrane (F). Bars, 10 μm.

Fig. 5: Electron micrographs of cryosections of electric organ. (A) Tissue section showing an elongated nerve terminal cut in longitudinal section, double-labeled with 2C-9 and CT, and visualized with 5- and 10-nm colloidal gold-conjugated secondary antibodies, respectively. Arrows indicate 10-nm gold found only in the invaginations of the postsynaptic membrane and in the synaptic cleft, while arrowheads indicate 5-nm gold found in the invaginations of the postsynaptic membrane, in the synaptic cleft, and on the nonsynaptic surface of the nerve terminals. (B) Tissue section showing elongated nerve terminals cut in cross section, labeled with 4E-7, and visualized with 5-nm gold. Arrowheads indicate the 5-nm gold found selectively on the nonsynaptic surface of the nerve terminals. Bars, 1 μm.

Having completed sequencing of most of the tryptic peptides and a large number of the CNBr peptides, it was relatively easy to conduct a separate analysis of the cysteine-containing peptides in the unreduced peptide to assign intra- and intersubunit disulfide bonds. The critical relevant sequences have been published in the concluding progress report, July 1, 1987, and in references 16, 19 and 20. These studies enabled us to show that the enzyme contains three intrasubunit disulfide loops, that Cys 231 was partially reduced, and that Cys 572 formed the intersubunit disulfide between homologous subunits. Since that time, we have been working on the intersubunit disulfide that connects the head and tail subunits and have evidence for the involvement of Cys 231. This comes from analysis of CNBr peptides and constitutes one of the studies we propose to continue.

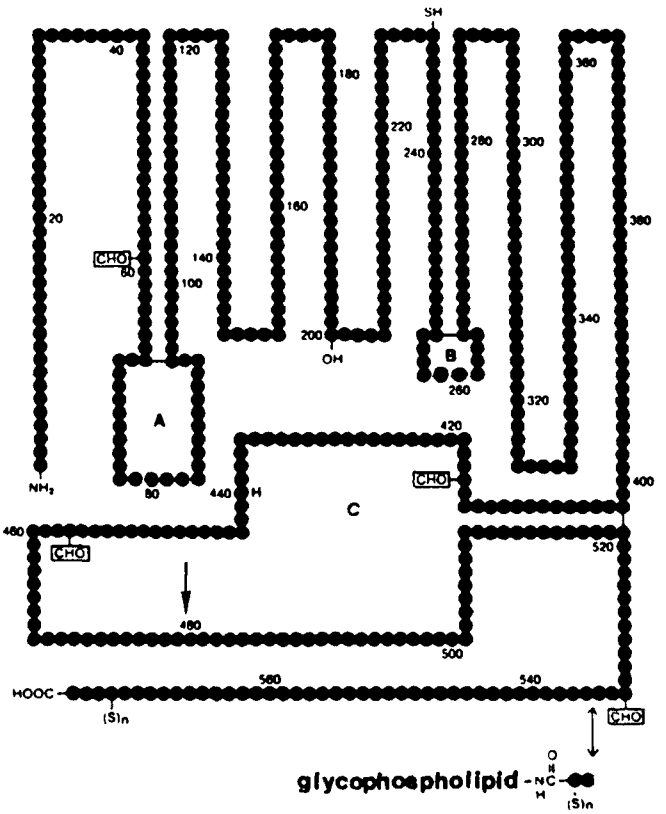

Fig. 6: Secondary Structures of Torpedo Acetylcholinesterases Based on Disulfide Bond Assignments (19). The active-site serine is shown at position 200, the free cysteine at 231 and the catalytic histidine at 440. The three disulfide loops Cys 67 to 94, Cys 254 to Cys 265, Cys 402 to Cys 511, are designated as A,B,C. Cys 572 bonds in intersubunit linkages. The single arrows after 479 denotes the exon 1-2 junction while the double arrow after 535 denotes the exon-intron junction of alternative mRNA processing.

III. CONCLUSIONS

Structural Divergence in the Acetylcholinesterase Species

Sequencing of the catalytic subunits from the asymmetric and hydrophobic forms of acetylcholinesterase showed that the two enzymes diverge at residue 534 and yield the sequence shown below:

Asymmetric: LLNATETIDEAERQWKTEFHRWSSYMMHWKNQFDHYSRHESCAEL
Hydrophobic: LLNATAC

The sequence of the asymmetric form was determined in studies on the previous contract. To obtain the LLNATAC sequence, we removed the acyl chains of the glycophospholipid by phospholipase C digestion. CNBr digestion yielded the deacylated peptide containing the glycan-inositol which could be isolated by size-fractionation followed by reverse phase high pressure liquid chromatography. Gas-phase sequencing yielded the above sequence. Ethanolamine and glucosamine could be detected upon amino acid analysis. This peptide was found in four peaks, all of which yielded the same peptide sequence but different posttranslational modifications.

Subsequent work with RNAse protection experiments[21] and then with genomic cloning[23] showed that the open reading frame of the enzyme is encoded by two constant exons, base -22 to 1502 and base 1503 - 1669. Base 1 is defined by the ATG start site in the leader peptide). The third exon starting at base 1670 is alternatively spliced and gives rise to two forms of acetylcholinesterase described above. Assignment to exon-intron boundaries shows the splice site to correspond to the appropriate position in the amino acid sequence[17,19]. Confirmation of the splicing mechanism has also come from loop-out mutagenesis experiments where we have been able to express the hydrophobic form of acetylcholinesterase by constructing a cDNA from the genomic clone by loop-out of the intron.

Studies of Antibody Interactions with Acetylcholinesterase - These studies have three primary directions. The first is to examine surface accessibility of various domains of the molecule. This work is being done in collaboration with B.P. Doctor WRAIR. We found that the active center of the enzyme must reside within the cleft, since monoclonal and polyclonal antibodies prepared to the active center peptide react only following denaturation. By contrast, antibodies directed to a C-terminal carboxy peptide react equally well with both the native and denatured enzyme.

The second endeavor identified epitopes for specified monoclonal antibodies. Epitopes for 2C-9 and 4E-7 have been localized to a CNBr peptide between residues 46 and 62 and we hope to narrow down epitope identification even more in the future. This is particularly important for 4E-7, since it reacts with an unusual posttranslational modification, which is found only on the hydrophobic enzyme species.

The third endeavor was to employ antibodies that distinguish the molecular forms of acetylcholinesterase in order to examine differential localization in the synapse. These studies show the asymmetric form of the enzyme to exist deep in the postsynaptic invaginations, while the hydrophobic form shows a punctate distribution, a position more removed from the postsynaptic membrane and a localization around presynaptic nerve endings.

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